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Increased Oxidative DNA Damage in *Helicobacter pylori*-infected Human Gastric Mucosa[1]

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ABSTRACT

***Helicobacter pylori* causes type B gastritis. It shows strong association with the development of gastric carcinoma. A plausible hypothesis for the missing link between *H. pylori* infection and gastric carcinogenesis involves oxygen free radical-induced DNA damage. To test this hypothesis, we compared the amount of 8-hydroxydeoxyguanosine, a marker for oxygen free radical-induced DNA damage, in the DNA of human gastric mucosa with and without *H. pylori* infection. Gastric antral biopsies were taken from pediatric patients and volunteers to select *H. pylori*-positive and *H. pylori*-negative specimens. The 8-hydroxydeoxyguanosine content of the gastric mucosal DNA was measured after *H. pylori*-positive and *H. pylori*-negative volunteers were identified. The increased level of oxidative DNA damage suggests the mechanistic link between *H. pylori* infection and gastric carcinoma.**

INTRODUCTION

Helicobacter pylori causes type B chronic gastritis (1), a long-standing and possibly lifelong gastritis, if it is not eradicated by a specific anti-*H. pylori* chemotherapy (2). This chronic gastritis has been shown to be sequentially followed by chronic atrophic gastritis, intestinal metaplasia, dysplasia, and carcinoma in a susceptible population (3), and, recently, epidemiological reports have suggested that *H. pylori* might be a possible major determinant in the carcinogenesis of gastric cancer (4–7).

Seroepidemiological and endoscopic studies revealed that most Korean people became carriers of *H. pylori* from early childhood (8, 9). Gastric cancer is the most common cancer in Korea. The annual incidence of gastric cancer in 1986–1987 was estimated as 57.9 and 25.1/100,000 for males and females, respectively, one of the highest values in the world (10). However, the mechanistic link between the *H. pylori* infection and gastric carcinogenesis is still hypothetical.

H. pylori infection induces active inflammation with neutrophilic infiltration and also elicits chronic inflammation with infiltration of lymphocytes, macrophages/monocytes, and plasma cells in the lamina propria of the mucosa of human gastric antrum, with the characteristic patchy distribution of the lesions (11–14). These neutrophils and macrophages/monocytes produce oxygen free radicals that could cause DNA damage to the adjacent cells (15). The DNA damage by oxygen free radicals could have harmful consequences, leading to gene modifications that are potentially mutagenic or carcinogenic. This long-standing and lifelong active and chronic inflammation of the gastric mucosa by *H. pylori* might override the human body's natural ability to repair the DNA damage of the rapidly proliferating epithelial stem cells of the gastric glands.

Recently, 8-OH-dG[3] has become accepted as a sensitive marker for oxidative DNA damage (16, 17). There have also been several observations that it could be mutagenic during DNA replication (18, 19) and carcinogenic *in vivo* (20, 21).

In this study, we measured the 8-OH-dG content of DNA from human gastric mucosa with and without *H. pylori* infection to show that increased DNA damage is associated with *H. pylori* infection.

MATERIALS AND METHODS

Selection of Volunteers. This study was performed in accordance with the Declaration of Helsinki and was approved by the Ethics Committee at Gyeongsang National University Hospital. All volunteers gave informed written consent. The volunteers were recruited from the children who underwent endoscopy at the Department of Pediatrics due to abdominal complaints from July 1992 to October 1993.

We selected *H. pylori*-positive and *H. pylori*-negative volunteers to compare the 8-OH-dG content of the gastric mucosal DNA of each group. *H. pylori*-positive volunteers were defined when all of the gastric biopsy specimens showed evidence of *H. pylori* infection by culture and urease test, and when the serum of the volunteer also showed a positive result in immunoblot analysis. *H. pylori*-negative volunteers were defined when none of the gastric biopsy specimens of the volunteer showed any evidence of *H. pylori* infection by culture, urease test, and histopathological methods as well as when the serum of the volunteer was not reactive in immunoblot analysis.

Endoscopic Biopsy. Endoscopy was performed using an Olympus XP-20 gastroscope by the same endoscopist. Seven pieces of biopsy specimens were taken from the gastric antrum of the individual participant. The first two samples were sent to a microbiology laboratory for culture and urease test within 1 h of collection. Another two samples were sent for routine histology and Warthin-Starry silver staining. The remaining three samples were immediately frozen and kept at −70°C until the 8-OH-dG was measured.

Bacterial Culture and Urease Testing. Two pieces of the biopsy specimens were inoculated by smearing onto a Mueller-Hinton agar plate containing 10% bovine serum, vancomycin (6.9 μM/liter), nalidixic acid (108 μM/liter), and amphotericin B (1.1 μM/liter) for bacterial culture, and then immediately inoculated into 2% urea plus phenol red-buffered solution for urease test. The plate was incubated at 37°C under 10% CO_2 and 100% humid atmosphere for 7 days. The bacteria were identified as *H. pylori* on the basis of their morphology and urease production. The urease test was considered as positive when the urea solution changed color from yellow to pink at 37°C within 48 h.

Histopathological Analysis. Two pieces of the biopsy specimens were fixed in 10% buffered formalin, embedded in paraffin, and sectioned. Tissue slides were stained with H&E for routine histology, and Warthin-Starry silver stain was used for the detection of *H. pylori*. Grading of the chronic gastritis, neutrophilic infiltration, and the density of *H. pylori*-like organisms was conducted as suggested by the Sydney System: 0, absent; 1, mild; 2, moderate; and 3, severe (14).

Immunoblot Analysis. Sera were collected from the volunteers when the endoscopy was performed and were screened for anti-*H. pylori* antibody using immunoblot analysis as described by Youn *et al.* (22). Whole-cell lysate of *H. pylori* was separated by 10–20% gradient SDS-PAGE and then transferred onto a nitrocellulose filter. The blot was incubated with a 1:5 dilution of serum and subsequently labeled with alkaline phosphatase-conjugated goat antihu-

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[3] The abbreviations used are: 8-OH-dG, 8-hydroxydeoxyguanosine; dG, deoxyguanosine.

Table 1 *Individual data of bacteriological, serological, and histopathological examinations and 8-OH-dG measurement of H. pylori-positive and H. pylori-negative volunteers*

Identification no. of participants	Age (yr)	Sex	Microbiological examination			Histopathological examination[a]			8-OH-dG content[b]
			Culture	Urease	Immunoblot	Lymphocytic infiltration	Neutrophilic infiltration	*H. pylori* density	
1	2.25	M[c]	+	+	+	1	0	0	0
2	2.66	M	+	+	+	1	0	0	0
3	4.25	M	+	+	+	2	1	1	11
4	4.50	M	+	+	+	1	0	0	31
5	4.50	M	+	+	+	1	0	0	0
6	4.75	M	+	+	+	0	0	0	25
7	4.75	M	+	+	+	1	0	0	4
8	5.84	M	+	+	+	1	0	0	4
9	6.84	M	+	+	+	3	2	3	42
10	7.58	M	+	+	+	2	1	1	5
11	7.84	M	+	+	+	1	0	0	4
12	8.33	M	+	+	+	2	0	0	18
13	8.66	M	+	+	+	3	2	3	3
14	9.00	M	+	+	+	2	1	1	2
15	9.50	M	+	+	+	2	0	0	4
16	10.00	M	+	+	+	2	1	1	5
17	10.33	M	+	+	+	3	1	0	24
18	10.50	M	+	+	+	2	1	2	2
19	10.75	M	+	+	+	1	0	0	10
20	11.33	M	+	+	+	3	0	1	46
21	11.66	M	+	+	+	3	1	2	13
22	11.58	M	+	+	+	2	0	2	5
23	13.00	M	+	+	+	3	0	1	0
24	13.00	M	+	+	+	2	0	3	5
25	13.84	M	+	+	+	3	1	1	31
26	14.58	M	+	+	+	3	1	2	22
27	15.00	M	+	+	+	3	2	1	5
28	16.00	M	+	+	+	3	0	1	11
29	16.50	M	+	+	+	3	1	2	43
30	3.08	F	+	+	+	2	0	0	4
31	3.75	F	+	+	+	1	0	0	27
32	4.25	F	+	+	+	2	2	0	3
33	7.75	F	+	+	+	0	0	0	0
34	8.16	F	+	+	+	3	1	1	25
35	8.84	F	+	+	+	3	1	2	46
36	9.00	F	+	+	+	1	0	0	10
37	9.00	F	+	+	+	1	0	0	3
38	10.58	F	+	+	+	3	1	1	2
39	11.25	F	+	+	+	2	0	0	30
40	12.00	F	+	+	+	1	0	0	14
41	12.25	F	+	+	+	1	0	0	4
42	12.25	F	+	+	+	3	2	3	37
43	12.50	F	+	+	+	3	1	1	4
44	12.58	F	+	+	+	2	1	1	23
45	12.92	F	+	+	+	3	3	0	0
46	12.92	F	+	+	+	2	1	1	0
47	13.00	F	+	+	+	2	1	2	2
48	13.00	F	+	+	+	2	0	0	0
49	14.66	F	+	+	+	3	0	2	0
50	16.92	F	+	+	+	3	1	3	21
51	0.84	M	−	−	−	1	0	0	3
52	1.16	M	−	−	−	1	0	0	0
53	1.84	M	−	−	−	0	0	0	6
54	2.16	M	−	−	−	1	0	0	2
55	2.50	M	−	−	−	1	0	0	8
56	2.75	M	−	−	−	1	0	0	5
57	3.75	M	−	−	−	1	0	0	0
58	6.16	M	−	−	−	1	0	0	10
59	6.84	M	−	−	−	1	0	0	0
60	8.84	M	−	−	−	0	0	0	24
61	10.00	M	−	−	−	2	0	0	3
62	10.50	M	−	−	−	1	0	0	4
63	12.40	M	−	−	−	1	0	0	3
64	13.84	M	−	−	−	1	0	0	0
65	15.08	M	−	−	−	1	0	0	0
66	2.75	F	−	−	−	0	0	0	2
67	3.00	F	−	−	−	1	0	0	18
68	3.16	F	−	−	−	1	0	0	0
69	3.25	F	−	−	−	1	0	0	3
70	4.40	F	−	−	−	0	0	0	1
71	7.00	F	−	−	−	1	0	0	13
72	11.00	F	−	−	−	1	0	0	3
73	12.00	F	−	−	−	1	0	0	9
74	14.00	F	−	−	−	1	0	0	14

[a] Grade 0, absent; grade 1, mild; grade 2, moderate; grade 3, severe (graded according to the Sydney System).
[b] Number of 8-OH-dG residues/10^5 dG.
[c] M, male; F, female.

man IgG (Promega) before staining with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. The presence of a M_r 120,000 protein band was considered seropositive.

Measurement of 8-OH-dG Content. DNA isolation was carried out as described by Kasai *et al.* (20). In brief, frozen gastric mucosal biopsy specimens were homogenized (three to four strokes) in a precooled Teflon homogenizer. Oxygen was removed from all of the tubes and buffers by argon gas prior to homogenization. DNA was isolated according to the procedure described by Marmur (23), with a slight modification (20). The isolated DNA was stocked in the dried state under argon at 5°C until 8-OH-dG analysis. DNA was digested to deoxynucleosides by treatment with nuclease P1, then with *Escherichia coli* alkaline phosphatase, and analyzed by a high-performance liquid chromatography-electrochemical detection system.

The content of guanine in the DNA digest was determined by UV detection. The UV detector was placed in line between the column and the electrochemical detector. 8-OH-dG content was expressed as the number of the residues per 10^5 dG.

Statistical Analysis. The Wilcoxon rank sum test was used for two group comparisons, the Kruskal-Wallis test for four group comparisons was used, and the Spearman rank correlation was used to test the association between two variables.

RESULTS

***H. pylori*-positive and -negative Volunteers.** The *H. pylori*-positive volunteers consisted of 29 males and 21 females (Table 1). Their median age was 10 years and ranged from 2 to 16 years. On the histopathological examinations, the lymphocytic infiltration was of the following grades: absent, 2 (4%); mild, 13 (26%); moderate, 16 (32%); and severe, 19 (38%), the neutrophilic infiltration was of these grades: absent, 26 (52%); mild, 18 (36%); moderate, 5 (10%); and severe, 1 (2%); and *H. pylori* density was of these grades: absent, 23 (46%); mild, 14 (28%); moderate, 8 (16%); and severe, 5 (10%; Table 2).

Spearman's correlation coefficients between the grades of histopathological variables of gastritis of the 50 *H. pylori*-positive specimens were 0.69 ($P = 0.0001$) between *H. pylori* density and lymphocytic infiltration, 0.56 ($P = 0.0001$) between *H. pylori* density and neutrophilic infiltration, and 0.63 ($P = 0.0001$) between lymphocytic and neutrophilic infiltrations.

The *H. pylori*-negative volunteers consisted of 15 males and 9 females (Table 1). Their median age was 6 years and ranged from 1 to 15 years. The gastric mucosal biopsy specimens, which did not show any *H. pylori*-like organisms, and neutrophilic infiltrations in the histopathological examination were selected as *H. pylori*-negative control. Lymphocytic infiltrations of grades moderate, 1 (4.4%), and mild, 19 (79.1%), were present (Table 2).

There was no differences in age and sex between *H. pylori*-positive and -negative volunteers (Table 2).

Table 2 *Summary of bacteriological, serological, and histopathological examinations of H. pylori-positive and H. pylori-negative volunteers*

Variable	*H. pylori*-positive group	*H. pylori*-negative group
No. of volunteers	50	24
Sex (M[a]/F)	29/21	16/9
Median age, yr	10	6
(range)	(2–16)	(1–15)
Culture	+	−
Urease test	+	−
Immunoblot analysis	+	−
Histology[b]		
Chronic gastritis (grade 0/1/2/3)	2/13/16/19	4/19/1/0
Neutrophilic infiltration (grade 0/1/2/3)	26/18/5/1	24/0/0/0
Density of *H. pylori* (grade 0/1/2/3)	23/14/8/5	24/0/0/0

[a] M, male; F, female.
[b] Grade 0, absent; grade 1, mild; grade 2, moderate; grade 3, severe (graded according to the Sydney System).

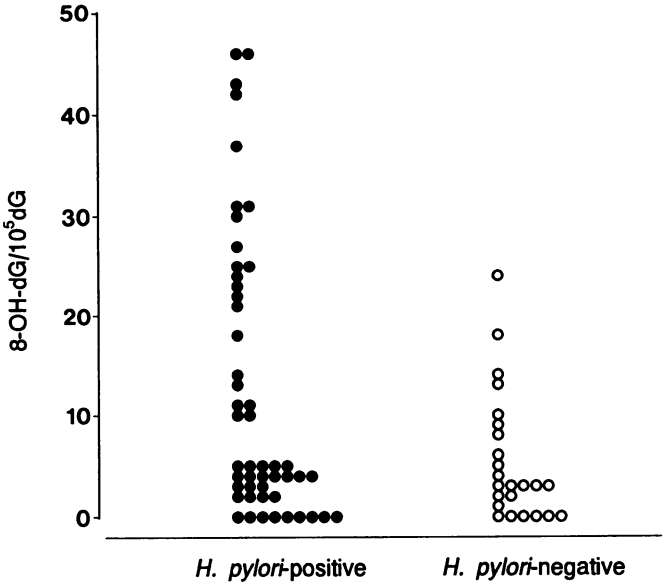

Fig. 1. Distribution of 8-OH-dG content of gastric mucosal DNA according to the *H. pylori* state. Mean values of 8-OH-dG content of the gastric mucosal DNA of *H. pylori*-positive volunteers (●) and *H. pylori*-negative volunteers (○) were 12.60, with a SE of 1.97, and 5.45, with a SE of 1.29, respectively.

Comparison of 8-OH-dG Content between the *H. pylori*-positive and -negative Volunteers. 8-OH-dG content of gastric mucosal DNA of the *H. pylori*-positive volunteers ranged from 0 to 46 8-OH-dG/10^5 dG, and the mean value of 8-OH-dG content was 12.60, with a SE of 1.97. 8-OH-dG content of gastric mucosal DNA of the *H. pylori*-negative volunteers ranged from 0 to 24 8-OH-dG/10^5 dG, and the mean value of 8-OH-dG content was 5.45, with a SE of 1.29 (Fig. 1). Gastric mucosal DNA of *H. pylori*-positive volunteers contained 2.3-fold higher levels of 8-OH-dG than those of *H. pylori*-negative volunteers ($P = 0.049$). There was no difference in the 8-OH-dG content between males and females (Table 1) and no correlation between age and 8-OH-dG content irrespective of *H. pylori* infection (Fig. 2).

Correlation of the 8-OH-dG content with any histopathological variables of *H. pylori* infection was not significant, and the Kruskal-Wallis test for the comparison of 8-OH-dG contents among the grades of each histopathological variables was not significant.

These data indicate that *H. pylori* infection itself rather than the histopathological variables was a major determining factor for the increased 8-OH-dG content in the gastric mucosa.

DISCUSSION

According to the seroepidemiological study (8) using immunoblot analysis done in Korea, *H. pylori* infection begins in infancy. The infection rate reaches 50% at 5 years of age and maintains 80–90% after 8 years of age. These findings were consistent with the endoscopic mucosal biopsy study performed on 48 adult healthy volunteers, which revealed that 45 (90%) of them were carriers of *H. pylori* (9). Therefore, one of the most difficult problems we encountered in this study was to obtain gastric mucosal biopsy specimens free from *H. pylori* infection, even from children. More strict criteria were used to define the *H. pylori*-negative than the *H. pylori*-positive volunteers as mentioned in "Materials and Methods." In defining the *H. pylori*-positive volunteers, a difficult problem we also met was the fact that the biopsy specimens for 8-OH-dG measurement, bacteriological examination, and histopathological examination are unavoidably different. It should be noted that the type B gastritis shows a typical pattern in the distribution of the lesion, which is multifocal in the human

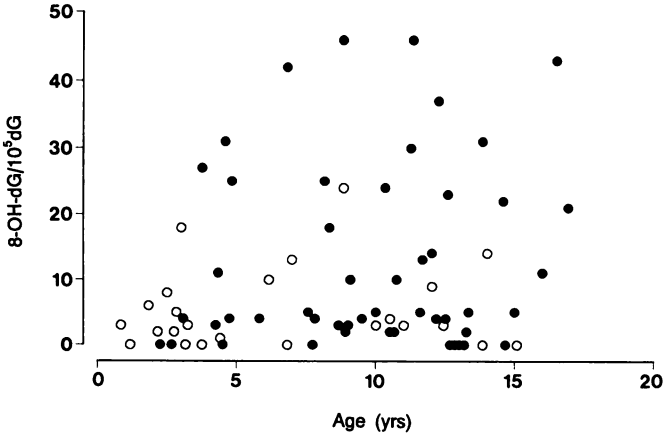

Fig. 2. Distribution of 8-OH-dG content of gastric mucosal DNA according to age. ●, *H. pylori* positive; ○, *H. pylori* negative.

gastric antrum in the early phase of infection (11–14). The observation that 23 *H. pylori*-positive volunteers did not show *H. pylori*-like organisms in Warthin-Starry silver stain (Tables 1 and 2) indicates the inconsistencies among the biopsy specimens from the same *H. pylori*-positive individual. These facts suggest that *H. pylori*-negative biopsy specimens might exist in the *H. pylori*-positive group as well as the *H. pylori*-positive specimens in the *H. pylori*-negative group. Although this study was performed within this kind of inherent methodological limitations, the 8-OH-dG content of gastric mucosal DNA of the *H. pylori*-positive group was shown to be 2.3-fold raised when compared to that of the *H. pylori*-negative group.

Our finding of higher levels of oxidative DNA damage in the gastric mucosa during the early phase of *H. pylori* infection supports the hypothesis that the oxygen free radicals persistently produced in the gastric mucosa due to *H. pylori* infection are the driving force that transforms the chronic gastritis ultimately into gastric carcinoma. If the major effect of oxygen free radicals on the rapidly proliferating gastric epithelial stem cells is cell death, atrophic gastritis would appear. Mutation on the DNA in the stem cells induced by free radical damage could lead to intestinal metaplasia, dysplasia, and gastric carcinoma in the long term. Cell death and mitogenesis have also been known as potent promoters for mutagenesis as well as carcinogenesis (24). However, we caution that our measurements were made on total DNA extracted from the biopsy specimens, and the validity of our hypothesis is dependent on the presence of increased levels of 8-OH-dG in the DNA of the stem cell of the glandular epithelium of the gastric mucosa.

Gastric and duodenal ulcers are associated with *H. pylori* infection (1). These gastroduodenal ulcers are also considered to be associated with enhanced oxygen free radical formation in the gastroduodenal mucosa (25–28). If free radical damage to DNA is an important factor in the chain of pathogenesis leading to gastric carcinoma, it is conceivable that administration of antioxidants may have preventive benefits. In fact, intake of a diet rich in vitamin C, a potent antioxidant, is known to inhibit gastric carcinogenesis in both case-controlled and prospective dietary surveys (29–32).

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